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Retroviral transduction with SOX9 enhances re-expression of the chondrocyte phenotype in passaged osteoarthritic human articular chondrocytes[1]

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Summary

Objectives: Articular chondrocytes proliferate in monolayer culture, but the expression of the transcription factor SOX9 falls and the ability of the cells to reform cartilage tissue declines. We have investigated whether retroviral SOX9 expression in extensively passaged human articular chondrocytes from osteoarthritic (OA) joints enables the cells to regain a cartilage matrix forming phenotype in pellet culture.

Design: Chondrocytes from normal and OA joints were retrovirally transduced with SOX9 and grown to passages 7–10 before being cultured as pellets of 500,000 cells for 14 days. Pellets were analysed by real time polymerase chain reaction, histology, immunohistochemistry and 1,9-dimethylmethylene blue assay.

Results: Chondrocytes from OA joints displayed higher expression of COL2A1 gene when transduced with SOX9 and cultured as pellets with 10% serum, but glycosaminoglycan (GAG) synthesis was low. Addition of transforming growth factor β-3 and insulin like growth factor-1 increased collagen II expression and GAG synthesis in these SOX9 transduced cell pellets. The cells adopted a rounded morphology and there was increased deposition of collagen II protein compared to control green fluorescent protein transduced cell pellets. Similar results were seen with transduced chondrocytes from OA or healthy cartilage. SOX9 transduced human dermal fibroblasts did not show any chondrogenic response.

Discussion: Transduction with SOX9 primed the passaged articular chondrocytes to regain a chondrocytic phenotype in pellet culture and to form a cartilaginous matrix, which was enhanced by growth factors. Following transduction, chondrocytes from OA joints showed a similar capacity for chondrogenic recovery as those from healthy joints, which suggested that OA does not permanently compromise the chondrocyte phenotype.

Key words: Human chondrocytes, Gene transfer, SOX9, Osteoarthritis, Articular cartilage.

Articular chondrocytes are the only cell type found within articular cartilage. They are responsible for the maintenance of the tissue's highly specialised extracellular matrix (ECM), in which the major components are type II collagen, aggrecan and hyaluronan. They are embedded in the ECM and it is established that their rounded morphology plays a critical role in the regulation of their phenotype[1]. As a result of this, the *in vitro* culture systems under which articular chondrocytes are grown have a large influence on the phenotype they express. In monolayer culture the cells spread, lose their round morphology and begin to proliferate. As the cell numbers expand, they appear fibroblastic and there is a loss of COL2A1 and aggrecan gene expression, and a concurrent up-regulation of COL1A1 expression. In contrast, in suspension culture over non-adherent plastic surfaces[2] or with cells encased in an agarose[1,3] or alginate gel[4], there is some retention of the ECM-producing phenotype and under these conditions the rate of proliferation is very slow.

Many studies have shown that monolayer expanded cells, which no longer synthesise appreciable cartilage ECM macromolecules, have a capacity to re-express the chondrocyte phenotype if they are placed in suspension culture[1]. However, with an increasing number of cell divisions the degree to which the phenotype can be regained decreases[5]. This is potentially a major hindrance to the use of expanded autologous articular chondrocytes in tissue engineering applications.

To promote the re-expression of the chondrocytic phenotype after repeated subculture of articular chondrocytes, various strategies have been employed. These include various types of three-dimensional (3D) culture, the use of media supplements such as hormones, cytokines, growth factors[6], variations in culture oxygen

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tension[7] and mechanical stimulation[8]. As an alternative approach, we have investigated the use of gene transfer and focussed on whether the chondrogenic potential of late passage human articular chondrocytes (HAC) is irreversibly lost and if this was affected by the disease status of the source cells, i.e. if they were from an osteoarthritic (OA) joint. Previous studies have demonstrated that introducing genes encoding growth factors known to enhance cartilage ECM production into chondrocytes can improve cartilage matrix synthesis by chondrocytes *in vitro* and *in vivo*[9–11]. In this study we have transduced SOX9, a transcription factor crucial for the induction and regulation of the chondrocyte phenotype[12], into monolayer expanded HAC from OA joints using a retroviral vector. We have previously shown that high efficiency retroviral transductions can be achieved by accelerating the growth of these cells using growth factors and that SOX9 transduction up-regulates COL2A1 gene expression in monolayer culture conditions[13]. In this study we have investigated if SOX9 transduced human OA articular chondrocytes after repeated passage can be induced to re-synthesise a cartilage matrix in pellet culture, and with growth factors. We also compared the recovery of phenotype by OA cells following transduction with SOX9 with that of chondrocytes from normal cartilage and contrasted the results with those from dermal fibroblasts that were similarly transduced.

Materials and methods

CHONDROCYTE ISOLATION, EXPANSION AND TRANSDUCTION

Human OA articular cartilage was obtained, with informed consent and local ethical committee approval, following total knee arthroplasties within 6 h of surgery. Tissue was taken from areas where the cartilage was largely intact from joints with clinical confirmation of degenerative OA. Human articular knee cartilage from non-OA joints was obtained from above knee amputation resulting from peripheral vascular disease. Tissue was carefully cut away from the underlying bone and the human OA or normal chondrocytes were isolated by a sequential trypsin/collagenase digestion and then grown as monolayers in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 100 units/ml penicillin and 100 units/ml streptomycin (all from Cambrex, Wokingham, UK). For efficient retroviral transduction, the rate of HAC growth was increased from passages 2–5 by the addition to the media of platelet derived growth factor-BB (PDGF-BB, 10 ng/ml), transforming growth factor (TGF) β-1 (1 ng/ml) and fibroblast growth factor-2 (FGF-2, 5 ng/ml) (all from Sigma, Poole, UK)[14]. The human OA or normal chondrocytes were transduced with a RKAT retrovirus containing a bicistronically expressed complementary deoxyribonucleic acid (cDNA) encoding human FLAG tagged SOX9 and green fluorescent protein (GFP), at a titre of 5×10^6 as has been described previously[13]. These are subsequently referred to as SOX9 transduced chondrocytes. For control of SOX9 transduction, chondrocytes were transduced with the RKAT vector containing only GFP and are referred to as GFP transduced chondrocytes. After repeated transductions (three times), the cells were at least 90% transduced (GFP positive). Normal human dermal fibroblasts (NHDF) (Cambrex, UK) were grown in DMEM, 10% FCS, 100 units/ml penicillin and 100 units/ml streptomycin and transduced with the same vectors using the same methodology as for the articular chondrocytes. The NHDF proliferated at a far greater rate than the HAC and were transduced without the growth factors present.

PELLET CULTURE

Transduced and non-transduced normal and OA chondrocytes were grown as cell pellets (500,000 cells/pellet) in DMEM, ITS1+ (Sigma, UK), 10 nM dexamethasone (Sigma, UK) and 25 μg/ml ascorbate 2-phosphate with or without 10% FCS. Pellets (500,000 cells) were formed with 1 ml medium in 15 ml conical culture tubes spun for 5 min at 160 g (swing out rotor). The wet weight of the cell pellets was recorded at the end of the culture period. The effect of growth factors on cell pellet cultures was assessed by supplementing the medium with 100 ng/ml insulin like growth factor-1 (IGF-1) and 10 ng/ml TGFβ-3 (both from Sigma, UK). All cultures were grown for 14 days in a humidified atmosphere with 5% CO_2 and medium was changed every 2–3 days. NHDF were cultured as pellets for 14 days in medium containing TGFβ-3 and IGF-1.

GENE EXPRESSION ANALYSIS

Total RNA was prepared from monolayer and pellet cultures using Tri Reagent (Sigma, UK). Pellet cultures were ground up in the Tri Reagent using Molecular Grinding Resin (Geno Technology Inc, St Louis, USA). cDNA was synthesised from 1 μg of total RNA using Moloney murine leukaemia virus reverse transcriptase and random hexamers (both from Promega, Southampton, UK) in a 25 μl reaction. Aliquots (1 μl) were amplified by polymerase chain reaction (PCR) in 25 μl reaction volumes on an ABI 7700 Sequence Detector or an MJ Research Opticon using a SYBR Green Core Kit (Eurogentec, Seraing, Belgium) with gene specific primers designed using ABI Primer Express software. Relative expression levels were normalised using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and calculated using the $2^{-\Delta Ct}$ method[15]. Primer sequences have been described previously for GAPDH, aggrecan, collagen I and collagen II[16] and SOX9[13]. Other primer sequences were as follows:

L-SOX5- Forward 5′–3′ ATCCCAACTACCATGGCAGCT, Reverse 5′–3′ TGCAGTTGGAGTGGGCCTA,

SOX6- Forward 5′–3′ GCAGTGATCAACATGTGGCCT, Reverse 5′–3′ CGCTGTCCCAGTCAGCATCT,

Deorin- Forward 5′–3′ CCTGGACACAACACCAAAAAGG, Reverse 5′–3′ ATCTGAAGGTGGATGGCTGTATCT,

Biglyan- Forward 5′–3′ CAGGAGGCGGTCCATAAGAAT, Reverse 5′–3′ ATGAGGAGGAGGAACAGAACATGT,

Fibromodulin- Forward 5′–3′ ACACCGTCCCCGATAGCTACT, Reverse 5′–3′ GAGGCCAGGCCATTGTTG.

All primers were from Invitrogen, Paisley, UK.

BIOCHEMICAL ANALYSIS OF PELLET CULTURES

After culture, pellets were digested overnight in 20 μl of 10 units/ml papain (Sigma, UK), 0.1 M sodium acetate, 2.4 mM ethylenediaminetetraacetic acid, 5 mM L-cysteine, pH 5.8 at 60°C. DNA content of the papain digest was

determined by measuring Hoechst 33258 dye (Sigma, UK) binding with a Hoeffer Dyna Quant 200 fluorometer. Glycosaminoglycans (GAG) were assayed in the papain digest using 1,9-dimethylmethylene blue (Aldrich, Poole UK)[17,18] with shark chondroitin sulphate (Sigma, UK) as standard.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

For histology, pellets were fixed in 4% formaldehyde and embedded in paraffin wax, 5 μm sections were cut and stained with 0.1% safranin-O. For immunohistochemical analysis, sections were digested with chondroitinase ABC and then incubated with antibodies to collagen I (C-18 polyclonal) or collagen II (N-19 polyclonal) (both collagen antibodies from Santa Cruz Biotechnology, Santa Cruz, USA). Immunolocalised antigens were visualised with a biotin conjugated donkey anti-goat secondary antibody (Santa Cruz Biotechnology) and a streptavidin–horseradish peroxidase labelling kit using 3,3′-diaminobenzidine (Dako, Ely, UK). All sections were examined using a Zeiss Axiovision light microscope.

STATISTICAL ANALYSIS

Statistically significant differences between gene expression values of control and transduced pellet cultures in the initial serum-containing or serum free cultures were determined using unpaired *t* tests. Gene expression, wet weight and GAG content in pellet cultures grown in serum with growth factors was analysed by univariate analysis of variance with Tukey *post hoc* tests using SPSS software.

Results

HAC were isolated from the residual cartilage of OA joints. The cells were cultured in monolayer and the rate of cell division was increased by the addition of growth factors (PDGF-BB, TGFβ-1 and FGF-2), which permitted efficient transduction with retroviral vectors as previously described[13]. At passage 3–4, chondrocytes were transduced with at least 90% efficiency with a bicistronic retroviral vector containing SOX9 and GFP and control transduced cells were produced expressing GFP alone using the same vector. Further culture was without PDGF-BB, TGFβ-1 and FGF-2. In non-transduced OA (or normal) chondrocytes at passage 7–10, which corresponded to a 1000-fold increase in cell number, the cells appeared fibroblastic with low expression of cartilage matrix genes, such as COL2A1 (down 5000-fold), and much increased gene expression of COL1A1 (up 10-fold). The expression of SOX9 was reduced to 10% of that of chondrocytes in articular cartilage, or at first passage in monolayer culture. The retroviral transduction with SOX9 did not result in over-expression, but restored SOX9 messenger RNA (mRNA) expression to levels comparable to those seen in freshly isolated chondrocytes.

The effects of SOX9 transduction on the cells' potential to recover a matrix forming chondrocyte phenotype was investigated in pellet culture, which has previously been shown to provide chondrogenic signals for differentiating chondrocytes[6]. Chondrocytes from late OA knee cartilage were specifically used in this study to understand if the joints pathology was an impediment to their use as chondrocytes for cell based approaches to cartilage repair. Chondrocytes were also obtained from non-OA joints. This allowed a comparison of the effects of SOX9 transduction on passaged chondrocytes from both healthy and diseased tissue. As a guide to chondrogenic phenotype, gene expression levels of COL1A1 and COL2A1 were determined by quantitative reverse transcription PCR analysis. Previous work showed that they provided a sensitive index of the chondrogenic status of chondrocytes, particularly as the level of expression of COL2A1 in passaged HAC was extremely low and less than 0.01% of that of cells in cartilage[13].

EFFECT OF SOX9 TRANSDUCTION ON PASSAGED HUMAN OA CHONDROCYTES

SOX9 transduction of OA chondrocytes caused a 10-fold increase in the expression of COL2A1 in monolayer cultures, compared to GFP transduced OA chondrocyte cultures [Fig. 1(A, B)]. The expression of COL2A1 in SOX9 transduced cells was further increased 10-fold after 14 days in pellet culture with serum. Without serum in pellet culture the increase in COL2A1 expression was smaller [Fig. 1(B)]. There were no effects of SOX9 transduction on COL1A1 gene expression, which remained very high. Without SOX9 transduction the OA chondrocytes showed a very poor chondrogenic response, as the GFP-OA control transduced cells showed a decrease in COL2A1 mRNA when grown for 14 days in pellet culture [Fig. 1(A)].

In order to assess ECM production in these cultures, the wet weight of pellets was compared. Pellets formed from SOX9 transduced OA chondrocytes after 14 days in serum free medium had double the wet weight of the GFP transduced OA chondrocyte pellets. Serum doubled the weight of SOX9 pellets, but had much less of an effect on GFP-control pellets [Fig. 1(C)]. FCS (10%) therefore caused greater stimulation of SOX9 transduced cells and resulted in a major increase in the accumulation of ECM. The pellets formed from SOX9 transduced cells were therefore 280% of the wet weight of pellets formed from GFP transduced OA chondrocytes.

EFFECT OF IGF-1 AND TGFβ-3 ON MATRIX ACCUMULATION IN SOX9 TRANSDUCED OA CHONDROCYTE PELLET CULTURES

As serum increased matrix accumulation, we also investigated the effects of IGF-1 and TGFβ, which are known to have anabolic effects on mature chondrocytes[19,20]. TGFβ-3 was used in these experiments as it is active in promoting chondrogenesis in mesenchymal stem cells[21]. Pellets of SOX9 transduced OA chondrocytes were treated in serum-containing medium supplemented with IGF-1 and TGFβ-3 at concentrations that are known to be effective in stimulating chondrocytes[21,22] (Figs. 2 and 3). IGF-1 had no effect on the wet weight of the SOX9 and GFP transduced OA cell pellets. Treatment with TGFβ-3 alone or with IGF-1 increased the wet weight of the SOX9 pellets by 61% and 68%, respectively. It also increased the wet weight of GFP transduced cell pellets by 65% and TGFβ-3 together with IGF-1 increased it by 123% [Fig. 2(A)]. The SOX9 transduced cell pellets with TGFβ3 and IGF-1 were thus 33% heavier than GFP transduced controls. GAG analysis showed that the growth factors caused a large increase in GAG accumulation [Fig. 2(B)]. In particular, IGF-1 together with TGFβ-3 showed a synergistic effect on GAG/DNA accumulation in SOX9 transduced OA cell pellets with an increase to 517% of that in pellets without growth factors. This was greater than the sum of the effects of TGFβ-3 or IGF-1 alone (increase relative to no-growth factor control

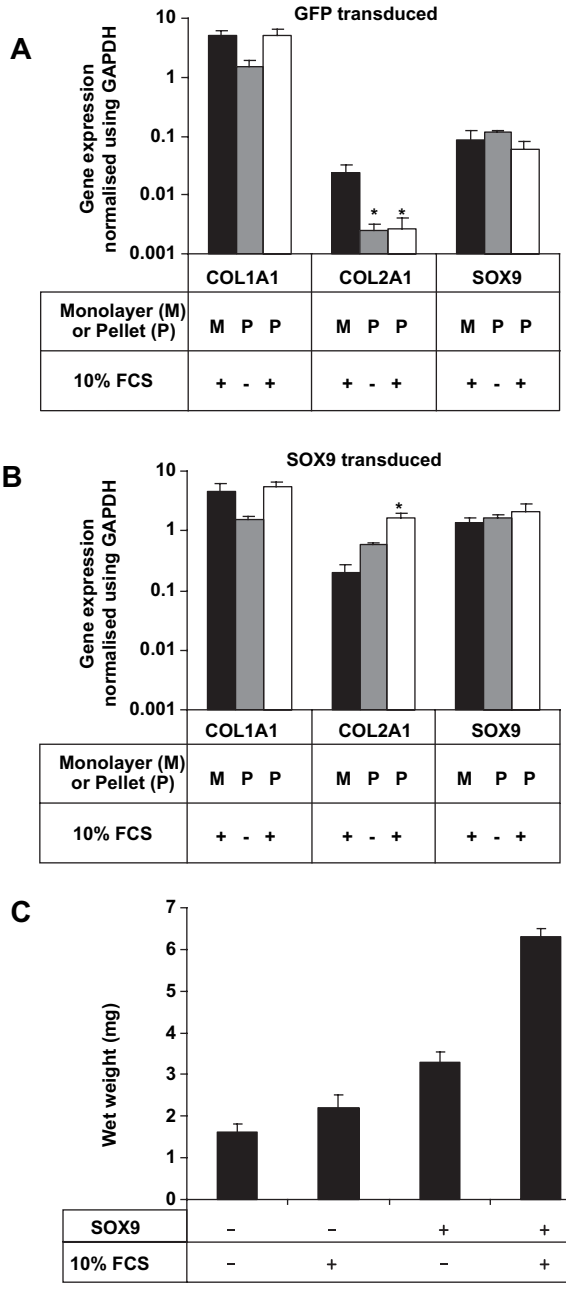

Fig. 1. (A) Real time PCR analysis of cDNA derived from monolayer and pellet cultures of untransduced human OA chondrocytes. The cells were examined between passages 7 and 10 as described in the text and pellet cultures were conducted for 14 days. Black bars represent expression levels for comparable monolayer cultures ($n = 11$), grey bars represent pellet cultures grown in serum free medium ($n = 4$) and white bars represent pellet cultures grown in medium containing 10% FCS ($n = 3$). * = $P < 0.05$ in pellet vs monolayer, Student's unpaired t test. (B) Real time PCR analysis of cDNA derived from monolayer and pellet cultures of human OA chondrocytes transduced with SOX9 retrovirus. The black bars represent expression levels for comparable monolayer cultures ($n = 9$), the grey bars represent pellet cultures grown in serum free medium ($n = 4$) and white bars represent pellet cultures grown in medium containing 10% FCS ($n = 3$). * = $P < 0.01$ in pellet vs monolayer, Student's unpaired t test. (C) Wet weight of GFP and SOX9 transduced human OA chondrocytes grown for 14 days as pellets in the presence or absence of 10% FCS. * = $P < 0.05$.

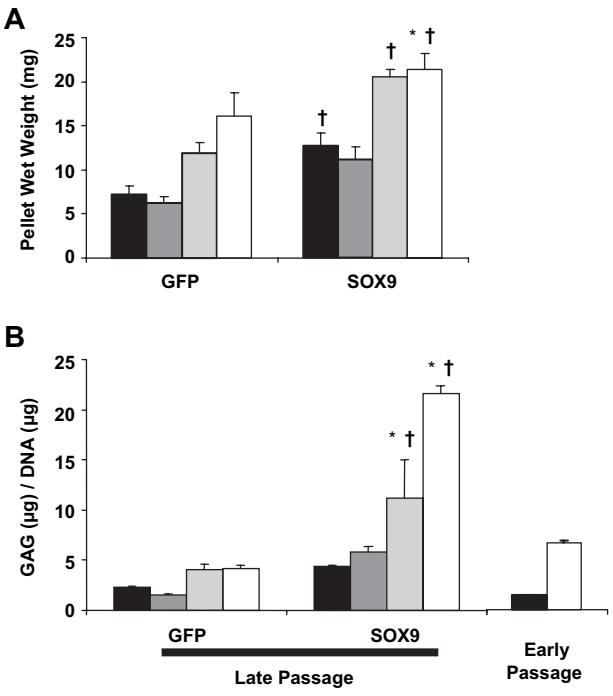

Fig. 2. Matrix accumulation in pellet cultures of late passage (passage 10) human OA chondrocytes transduced with SOX9 or GFP and early passage (passage 4) untransduced OA chondrocytes. Cultures were grown for 14 days in the presence or absence of growth factors. (A) Pellet wet weight, (B) GAG content of pellet normalised to DNA content (weight per weight). Pellets were grown without growth factors (black bars) or in the presence of 100 ng/ml IGF-1 (dark grey bars), 10 ng/ml TGFβ-3 (light grey bars) or both IGF-1 and TGFβ-3 together (white bars). * = $P < 0.05$ in growth factor treatment vs no growth factors, † = $P < 0.05$ in SOX9 transduced vs GFP transduced grown under the same conditions.

pellets of 253% and 132%, respectively). The effects of the growth factors on GAG accumulation in pellets of GFP transduced OA cells were much less than on SOX9 transduced OA cells. IGF-1 alone decreased the GAG per DNA (down 33%); TGFβ-3 increased it to 175% of the no-growth factor control and treatment with both growth factors gave results similar to those with TGFβ-3 alone. The overall GAG content of SOX9 transduced OA cell pellets after 14 days with both of the growth factors was thus five times more than in the pellets formed by GFP transduced OA cells under the same conditions and 10 times greater than in the pellets formed by GFP transduced OA cells without growth factors.

As the ability of chondrocytes to regain a matrix forming phenotype is reported to decline with passage in culture, some OA chondrocytes without retroviral transduction were tested at passage 4 to assess their natural chondrogenic response under similar culture conditions in pellets. This was also designed to provide a comparative measure of the chondrogenic response found in passage 7–10 SOX9 transduced OA chondrocytes. Pellets of passage 4 OA chondrocytes were formed and cultured for 14 days with or without TGFβ-3 and IGF-1 together. Without growth factors, the GAG/DNA content was slightly lower than in the late passage GFP transduced OA cells, but with TGFβ-3 and IGF-1 combined the GAG/DNA content increased to 459% of the no-growth factor control. This was a larger increase than that seen in the late passage GFP transduced cells

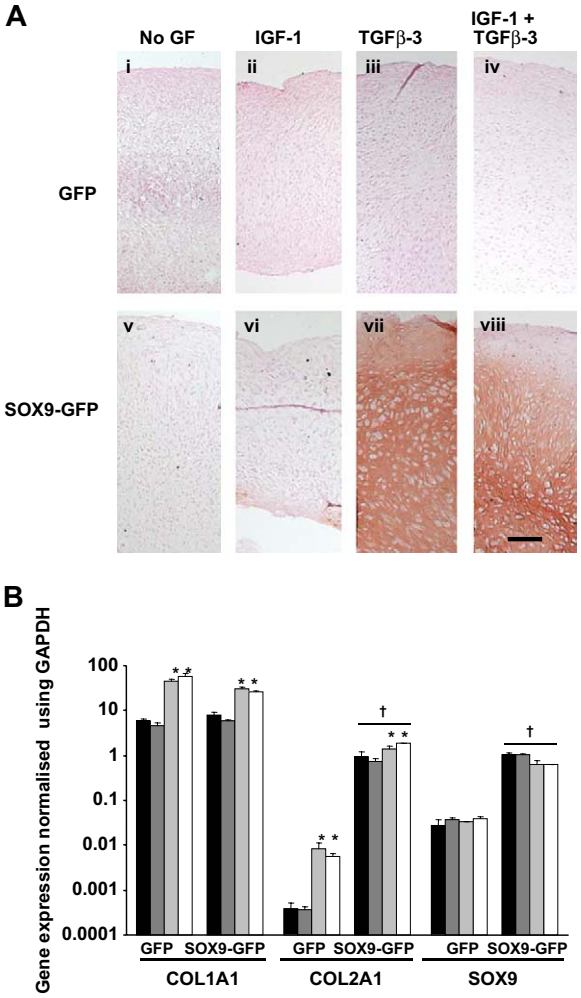

Fig. 3. (A) Histological analysis of SOX9 and GFP transduced late passage human OA chondrocytes grown as pellets for 14 days. Paraffin wax sections from pellets treated with or without growth factors were stained with safranin-O. GFP transduced (i–iv) and SOX9 transduced (v–viii) cell pellets were treated with no growth factor (i and v), 100 ng/ml IGF-1 (ii and vi), 10 ng/ml TGFβ-3 (iii and vii) or both IGF-1 and TGFβ-3 together (iv and viii). Scale bar = 100 μm. (B) Real time PCR analysis of cDNA derived from growth factor stimulated pellets of late passage OA HAC grown for 14 days. SOX9 transduced and GFP transduced cells were examined for mRNA levels of COL1A1, COL2A1 and SOX9. Pellets were grown without growth factors (black bars), in the presence of 100 ng/ml IGF-1 (dark grey bars), 10 ng/ml TGFβ-3 (light grey bars) or both IGF-1 and TGFβ-3 together (white bars). ($n = 3$ for all pellets). * = $P < 0.05$ in growth factor treatment vs no growth factors. † = $P < 0.05$ in SOX9 transduced vs GFP transduced grown under the same conditions.

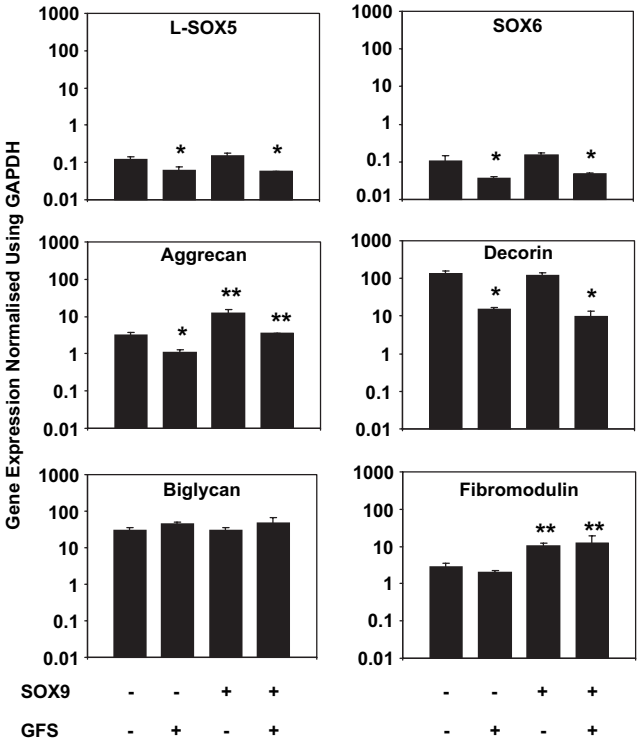

Fig. 4. Real time PCR analysis of cDNA derived from growth factor stimulated pellets of late passage human OA chondrocytes grown for 14 days. SOX9 and GFP transduced cells were examined for mRNA levels of L-SOX5, SOX6, aggrecan, decorin, biglycan, and fibromodulin as indicated. GFS indicates treatment with 100 ng/ml IGF-1 and 10 ng/ml TGFβ-3 together. * = $P < 0.05$ in growth factor treatment vs no growth factors. $n = 3$ in all instances except aggrecan and SOX6 analysis of GFP no-growth factor pellets ($n = 7$) and SOX9 no-growth factor pellets ($n = 5$) ** = $P < 0.05$ in SOX9 transduced vs GFP transduced grown under the same conditions.

[Fig. 2(B)]. However, the GAG/DNA was still less than that shown by SOX9 transduced OA chondrocytes at passage 10 grown under the same conditions.

Histochemical staining of the pellets with safranin-O showed that those formed from GFP transduced OA cells [Fig. 3(Ai– iv)] had low levels of poorly stained ECM. The SOX9 transduced OA cell pellets were also poorly stained in the absence of TGFβ-3 [Fig. 3(Av, vi)]. In contrast, they showed strong safranin-O staining with TGFβ-3 alone, or in combination with IGF-1 [Fig. 3(Avii, viii)]. In addition, the cells in these pellets had developed a more rounded morphology, characteristic of chondrocytes in cartilage. Analysis of gene expression showed that in SOX9 OA cell pellets, COL2A1 gene expression was much higher than in all control GFP transduced OA cell cultures and it was further stimulated by TGFβ-3, but not by IGF-1 [Fig. 3(B)]. SOX9 gene expression was at high levels in all of the SOX9 transduced cell cultures due to their transduction and it was unaltered by the growth factors. Both SOX9 transduced and GFP transduced OA cell pellets had similar high levels of COL1A1 mRNA and TGFβ-3 increased this expression. These results together with those of wet weight analysis and total GAG showed that the growth factors, chiefly TGFβ-3, greatly increased the chondrocytes' matrix forming phenotype.

EXPRESSION OF L-SOX5, SOX6 AND PROTEOGLYCAN GENES IN SOX9 TRANSDUCED OA CHONDROCYTE PELLET CULTURES TREATED WITH GROWTH FACTORS

To further characterise the effect of SOX9 transduction and growth factor stimulation (GFS) on OA chondrocyte pellet cultures we investigated the expression of L-SOX5 and SOX6 genes and also proteoglycans known to form part of the cartilage ECM (Fig. 4). L-SOX5 and SOX6 are homologous proteins which form either homo- or hetero-dimers and which bind to a chondrocyte specific enhancer

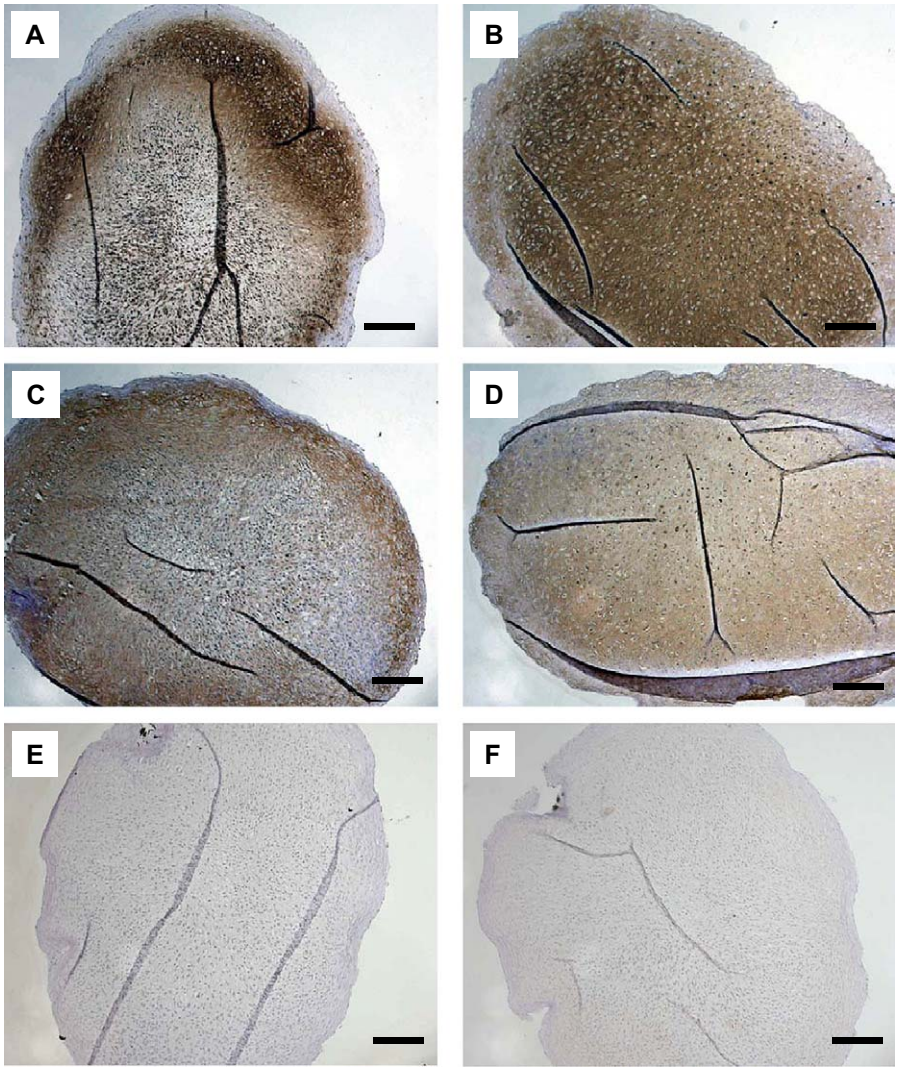

Fig. 5. Immunohistochemical analysis of 14-day OA chondrocyte pellet cultures grown in the presence of 100 ng/ml IGF-1 and 10 ng/ml TGFβ-3. Chondrocytes were transduced with GFP (A, C) or SOX9 (B, D). Sections were labelled with antibodies to collagen II (A and B) or collagen I (C and D). GFP transduced sections labelled using no primary antibody (E) or control IgG (F) are shown as examples of controls. Scale bars = 100 μm.

region within the first intron of the type II collagen gene in concert with SOX9[23]. Aggrecan is the major proteoglycan component of the ECM and decorin, biglycan and fibromodulin are small leucine rich proteoglycans that are all found within cartilage. Analysis of pellets showed that the presence of the growth factors decreased the expression of L-SOX5, SOX6 and decorin and this was independent of SOX9 transduction (Fig. 4). Biglycan expression was unchanged under any conditions and fibromodulin was up-regulated in SOX9 transduced cell cultures, but was unaffected by the growth factors. Aggrecan mRNA was higher in SOX9 transduced OA cells, but growth factors decreased its expression.

COLLAGEN DEPOSITION IN SOX9 TRANSDUCED OA CHONDROCYTE PELLET CULTURES TREATED WITH GROWTH FACTORS

To examine whether increased levels of COL2A1 gene expression translated into greater accumulation of collagen type II protein, SOX9 transduced OA chondrocyte pellets grown with TGFβ-3 and IGF-1 were immunostained with antibodies to collagen type II and collagen type I (Fig. 5). Pellets of SOX9 transduced OA cells were strongly labelled with anti-collagen type II, which was distributed uniformly throughout the pellet [Fig. 5(B)]. In contrast, in these pellets anti-collagen type I immunostaining was faint, but it was also evenly distributed [Fig. 5(D)]. In GFP transduced pellets, collagen type II was localised to an area just beneath the surface of the pellet, but there was much less present in the centre of the pellet [Fig. 5(A)]. Collagen type I was localised at the surface of the GFP transduced cell pellets corresponding to the collagen type II negative "rim" [Fig. 5(C)]. Primary antibody and non-specific IgG controls showed no staining [Fig. 5(E and F), respectively]. Therefore, SOX9 transduction of OA chondrocytes gave a more uniform collagenous network in pellet culture, which stained strongly for collagen type II and this may reflect a more uniform differentiated cell population throughout the pellet.

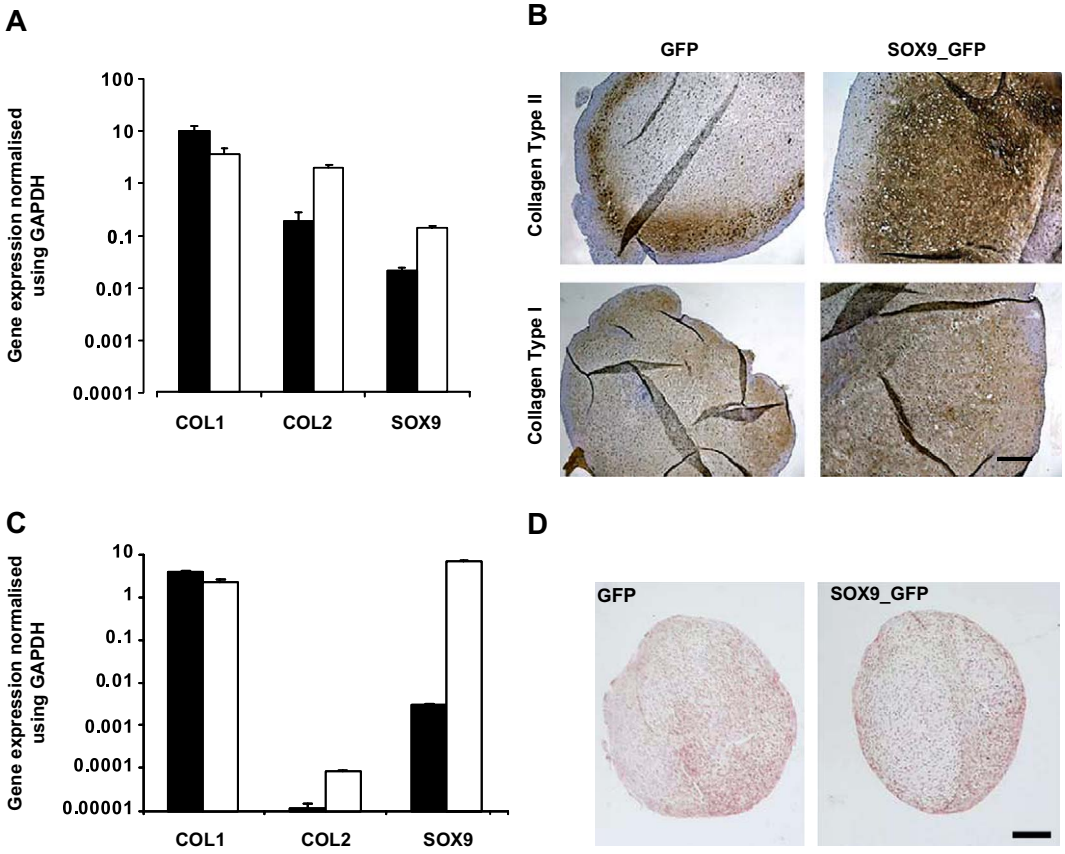

Fig. 6. Normal HAC or human dermal fibroblasts transduced with SOX9 and cultured as pellets for 14 days in media supplemented with 100 ng/ml IGF-1 and 10 ng/ml TGFβ-3. (A) Real time PCR analysis of pellets formed from normal chondrocytes, SOX9 transduced (white bars) and GFP transduced cells (black bars) were examined for mRNA levels for COL1A1, COL2A1 and SOX9. The scale is the same as that in Fig. 3 and the data are comparable to the OA chondrocyte expression levels shown in Fig. 3(B). (B) Immunohistochemical analysis of collagen type II and type I in 14-day normal chondrocyte pellet cultures, either GFP transduced or SOX9 transduced as indicated. Pellets were grown in the presence of 100 ng/ml IGF-1 and 10 ng/ml TGFβ-3. Scale bar = 100 μm. (C) Real time PCR analysis of 14-day NHDF pellet cultures transduced with GFP (black bars) or SOX9 (white bars) and grown in the presence of 100 ng/ml IGF-1 and 10 ng/ml TGFβ-3. (D) Safranin-O staining of sections from SOX9 or GFP transduced NHDF cell pellets grown for 14 days in the presence of 100 ng/ml IGF-1 and 10 ng/ml TGFβ-3. Scale bar = 100 μm.

SOX9 TRANSDUCTION AND PELLET CULTURE OF NORMAL HAC AND PRIMARY HUMAN SKIN FIBROBLASTS

In order to understand if OA disease status was a factor limiting the ability of articular chondrocytes to regain a matrix forming phenotype, experiments were set up to compare chondrocytes from OA and non-OA healthy joints. Chondrocytes from normal articular cartilage in non-OA joints were transduced with SOX9 and grown to late passage using the same technique as that used for the OA chondrocytes. Pellet cultures grown in combined TGFβ-3 and IGF-1 medium showed the same major increases in matrix deposition in pellet culture as those seen with the OA cells. Gene expression analysis showed similar stimulation of COL2A1 gene (10 fold) by SOX9 as that seen in the OA cells [Fig. 6(A)]. As with the OA cells, the increased levels of SOX9 affected COL2A1 but not COL1A1 expression, which remained unchanged. It should be noted that COL2A1 expression was 30 times higher in GFP transduced normal chondrocyte pellets than in those formed from GFP transduced OA chondrocytes at similar passage [comparing Fig. 6(A) to Fig. 3(B)]. The expression of COL1A1 was also high and of similar level to that found in pellets formed from SOX9 or GFP transduced OA chondrocytes. Immunohistochemistry for collagen type II and collagen type I in SOX9 and GFP transduced normal chondrocyte cell pellets showed a similar distribution to that in transduced OA chondrocyte pellets [Fig. 6(B)]. SOX9 transduction therefore enhanced the chondrogenic response to pellet culture and growth factors of both OA and normal chondrocytes. These results showed that the response of passaged articular chondrocytes to retroviral transduction with SOX9 was similar whether they were from an OA joint, or from a normal healthy joint of comparable age.

To determine whether the effects of SOX9 transduction were specific to chondrocytes, human dermal fibroblasts were transduced with SOX9 after passage in culture and were grown as pellets with TGFβ-3 and IGF-1. Gene expression analysis of these pellets showed that there was an increase in COL2A1 gene expression in SOX9 transduced fibroblasts compared to GFP transduced cells [Fig. 6(C)], but the level remained more than 1000-fold less than in SOX9 transduced OA chondrocytes. Histological analysis of the pellets showed that SOX9 transduction did not result in pellet enlargement, cell rounding or deposition of GAG-rich ECM [Fig. 6(D)]. Therefore, SOX9 transduction was unable to cause the chondrogenic differentiation, or

matrix forming phenotype in primary human dermal fibroblasts.

Discussion

Articular chondrocytes lose their phenotype when isolated and grown in monolayer culture[24]. Whilst it has been shown that the phenotype can be regained during early passage by growing the cells in 3D culture systems, this ability was reported to diminish in late passage cells[5]. It has also been shown that the expression of many cartilage matrix genes, such as collagen type II, is reduced in monolayer culture and that the transcription factor SOX9 is also down-regulated[2,25–27]. In a previous study we demonstrated that retroviral expression of SOX9 in human OA articular chondrocytes leads to an up-regulation in expression of collagen type II gene and protein[13]. These data corresponded well with the results of transient SOX9 transduction in murine primary cells, and in a number of chondrocytic cell lines[23,26]. However, despite the SOX9 expression, the collagen II gene expression levels in monolayer cultures were still several orders of magnitude less than those seen in chondrocytes *in vivo*. In this study we investigated whether combining SOX9 transduction with a 3D culture was able to renew the capacity of late passage human OA articular chondrocytes to form cartilage matrix. Furthermore, we tested if there was a difference in the response of OA and normal chondrocytes under these culture conditions.

Comparing the gene expression of cartilage matrix genes in chondrocytes with or without SOX9 transduction and in different culture conditions has enabled us to describe a number of factors which contribute towards the development of a stronger matrix forming chondrocyte phenotype in these late passage cells. Without SOX9 transduction, the OA chondrocytes transduced with GFP actually displayed lower levels of collagen II mRNA when placed in pellet culture and there was no increase in SOX9 expression. The endogenous chondrogenic response of these passaged OA cells was therefore very low. The exact mechanism by which chondrocytes respond to pellet culture is unclear, but it may involve increased cell–cell interactions and an improved environment for localised paracrine signalling. SOX9 transduction clearly increased the chondrogenic response of these passaged OA chondrocytes in pellet culture, but without anabolic growth factors present, the mechanisms active in pellet culture appeared only able to support a partial recovery of the chondrocyte phenotype. The addition of serum was able to significantly increase the wet weight of the SOX9 transduced cell pellets as well as promote collagen II gene expression and GAG accumulation. This effect of serum is likely to be due to the growth factors it contains. However, other components in serum, such as fibronectin, which has demonstrated importance in cell–ECM signalling during early stages of chondrogenesis[28], may also be influential. The effect of additional growth factors, IGF-1 and TGFβ-3, was also potentiated by SOX9 transduction. When the growth factors were added together, the pellets formed with SOX9 transduced OA cells differed in a number of ways from those transduced with GFP alone. Firstly, SOX9 transduced cells produced the heaviest pellets with the highest level of COL2A1 expression and GAG content. In addition, collagen type II immunostained uniformly throughout the SOX9 pellets. Therefore, SOX9 transduction potentiated a more robust chondrogenic response throughout the pellet, whereas the control GFP transduced cell pellets showed a less complete and patchy chondrogenic response. Finally, the effects of the growth factors on GAG accumulation in the pellets were only synergistic with SOX9 transduced cells. It is clear that SOX9 enhances the chondrocytes' ability to respond to these growth factors, to serum and to pellet culture, although the underlying mechanisms are as yet unclear. SOX9 is known to regulate the expression of genes such as collagen types II[26], XI[29], and IX[30] and aggrecan[31], which all encode important cartilage matrix proteins. There is evidence that cellular attachment to collagen type II is able to modulate TGFβ signalling in articular chondrocytes[32]. Furthermore, chondrocytes cultured as chondrons, with their pericellular matrix still intact, show significant differences in their reactions to culture stimuli[33–35]. The formation of a pericellular matrix facilitated in pellet culture may be necessary for efficient matrix signalling to chondrocytes and for modulating the transduction of those signals into changes in gene expression. There is evidence that SOX9 has a role and influence on bone morphogenetic protein and sonic hedgehog signalling in mesenchymal progenitor cells[36,37] and the increased expression of SOX9 may thus change the way in which the chondrocytes transduce the growth factor and other matrix signals.

It was a notable feature of these passaged OA chondrocytes that the high expression of COL1A1 gene was not accompanied by high collagen I protein expression in monolayer or in pellet culture. Collagen I immunostaining was weak, but was similar in SOX9 and GFP transduced cell pellets. These results suggest that collagen type I protein expression is not directly correlated with the level of COL1A1 mRNA and that there is major control of collagen type I translation to produce protein. The result also shows that SOX9 does not play a role in controlling the gene expression, protein synthesis or secretion of collagen type I. In OA cartilage, chondrocytes express higher levels of COL1A1 *in vivo* than chondrocytes from normal cartilage[38], but the level of COL1A1 mRNA increases in all chondrocytes, when isolated and grown in monolayer culture. There is the possibility that the techniques used would detect COL1A1 antisense in the PCR reactions, which has been detected in chick embryo chondrocytes[39]. However, whatever the mechanism for control of type I collagen protein expression, it is clear that it remains low in these passaged OA cells in pellet culture.

SOX9 interacts with cartilage specific promoters in conjunction with a number of transcriptional co-activators, which include the CREB and its paralog p300[40] as well as L-SOX5 and SOX6[23]. We investigated the expression of L-SOX5 and SOX6 in 14-day SOX9 transduced cell pellets cultured with or without TGFβ-3 and IGF-1 and found that not only was their expression unaffected by SOX9 transduction, but that their expression levels were reduced upon culturing in the presence of the growth factors. This was a surprise, as these two genes have been shown to co-operate with SOX9 at the collagen type II specific enhancer within the first intron[23], and to be essential factors for cartilage formation during development[41]. There is also clear evidence that chondrocyte progression through endochondral ossification is adversely affected as the expression of these SOX genes is lost in heterozygous and null mouse embryos[42]. In the SOX9 transduced cell pellet system, however, in spite of the reduced levels of L-SOX5 and SOX6 there was a marked increase in proteoglycan synthesis and a small increase in COL2A1 expression and increased collagen type II immunostaining. These results suggest that the levels of L-SOX5 and SOX6

expressed in passaged OA chondrocytes are sufficient to act in promoting/enhancing matrix protein expression once the level of SOX9 is increased.

To further characterise the phenotype of the SOX9 transduced OA cells in pellet culture, gene expression analysis of four proteoglycan genes present in cartilage was conducted. Aggrecan gene expression was increased by SOX9 transduction and this correlated with increases in GAG content of the pellets. This result adds to the evidence that SOX9 is involved in the transcriptional regulation of the aggrecan gene[31]. Of the three small leucine rich proteoglycans examined, only fibromodulin mRNA was up-regulated by SOX9 expression and as such may represent a novel target for SOX9 transcriptional regulation. Biglycan expression was unaffected by any of the culture conditions, whilst decorin expression was reduced in the presence of the two growth factors. It is interesting that decorin should be down-regulated in the presence of TGFβ-3, as a previous study has shown that TGFβ-1 up-regulates decorin in canine cartilage explant culture[43]. These data, along with the high levels of COL1A1 seen in all pellet cultures examined, suggest that the SOX9 transduced cells after 14 days of pellet culture still have many differences in expression from mature chondrocytes in cartilage.

This study shows that passaged human OA chondrocytes even after extensive subculture retain an ability to respond to SOX9 transduction, whereas dermal fibroblasts transduced with SOX9 display no signs of chondrogenesis even when grown in pellet culture. It was previously shown that SOX9 expression did not induce COL2A1 expression in murine non-chondrocytic cell lines grown in monolayer culture[23]. This shows that SOX9 alone cannot confer a chondrogenic status and suggests that passaged chondrocytes, even though they appear fibroblastic and down-regulate many matrix genes, retained elements of their chondrocyte origin. These data show that SOX9 provides a priming role to passaged chondrocytes, enabling them to respond to chondrogenic stimuli and re-initiate a matrix forming phenotype. The loss of SOX9 expression by the chondrocytes during monolayer culture appears to lead to a progressive decline in the ability of the cells to re-express their phenotype when placed in pellet cultures. Because SOX9 expression declines gradually during passages[27] there may be sufficient production of the protein during early stages of chondrocyte culture (<4 passages) to support a response to chondrogenic culture conditions. However, in the cells that we have examined in this study (>7 passages) the expression of SOX9 reached a level 10-fold lower than that in differentiated chondrocytes. This appears to be too low to allow the re-initiation of a chondrogenic phenotype over the 14-day culture period. Retroviral expression of SOX9 raised the mRNA level to that seen in chondrocytes *in vivo* and this facilitated the re-expression of the chondrocyte phenotype when placed in pellet culture with growth factors (IGF-1 and TGFβ-3). It remains to be determined what physiological signals initiate and maintain SOX9 expression.

Finally, we have shown that the responses of normal human chondrocytes in these experiments were the same as the responses of chondrocytes from OA joints following SOX9 transduction. The initial status of the OA chondrocytes appeared therefore not to have resulted in permanent differences in the expanded progeny. This is a very important concept, which implies that the potential of cells from OA joints may not be permanently compromised by their pathological background and given appropriate signals they may regain a chondrogenic phenotype.

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References

1. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30(1):215–24.
2. Stokes DG, Liu G, Dharmavaram R, Hawkins D, Piera-Velazquez S, Jimenez SA. Regulation of type-II collagen gene expression during human chondrocyte de-differentiation and recovery of chondrocyte-specific phenotype in culture involves Sry-type high-mobility-group box (SOX) transcription factors. Biochem J 2001;360(Pt 2):461–70.
3. Kuettner KE, Pauli BU, Gall G, Memoli VA, Schenk RK. Synthesis of cartilage matrix by mammalian chondrocytes *in vitro*. I. Isolation, culture characteristics, and morphology. J Cell Biol 1982;93(3):743–50.
4. Guo JF, Jourdian GW, MacCallum DK. Culture and growth characteristics of chondrocytes encapsulated in alginate beads. Connect Tissue Res 1989;19(2–4): 277–97.
5. Zaucke F, Dinser R, Maurer P, Paulsson M. Cartilage oligomeric matrix protein (COMP) and collagen IX are sensitive markers for the differentiation state of articular primary chondrocytes. Biochem J 2001; 358(Pt 1):17–24.
6. Jakob M, Demarteau O, Schafer D, Hinterman B, Dick W, Heberer M, *et al*. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation *in vitro*. J Cell Biochem 2001;81(2):368–77.
7. Domm C, Schunke M, Christesen K, Kurz B. Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. Osteoarthritis Cartilage 2002;10(1):13–22.
8. Lee CR, Grodzinsky AJ, Spector M. Biosynthetic response of passaged chondrocytes in a type II collagen scaffold to mechanical compression. J Biomed Mater Res 2003;64A(3):560–9.
9. Shuler FD, Georgescu HI, Niyibizi C, Studer RK, Mi Z, Johnstone B, *et al*. Increased matrix synthesis following adenoviral transfer of a transforming growth factor beta1 gene into articular chondrocytes. J Orthop Res 2000;18(4):585–92.
10. Smith P, Shuler FD, Georgescu HI, Ghivizzani SC, Johnstone B, Niyibizi C, *et al*. Genetic enhancement of matrix synthesis by articular chondrocytes: comparison of different growth factor genes in the presence and absence of interleukin-1. Arthritis Rheum 2000; 43(5):1156–64.
11. Brower-Toland BD, Saxer RA, Goodrich LR, Mi Z, Robbins PD, Evans CH, *et al*. Direct adenovirus-mediated insulin-like growth factor I gene transfer enhances transplant chondrocyte function. Hum Gene Ther 2001;12(2):117–29.
12. de Crombrugghe B, Lefebvre V, Nakashima K. Regulatory mechanisms in the pathways of cartilage and bone formation. Curr Opin Cell Biol 2001;13(6):721–7.

13. Li Y, Tew SR, Russell AM, Gonzalez K, Hardingham TE, Hawkins RE. Transduction of human articular chondrocytes with adenoviral, retroviral and lentiviral vectors and the effects of enhanced expression of SOX9. Tissue Eng 2004;10(3/4):575–84.
14. Barbero A, Ploegert S, Heberer M, Martin I. Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthritis Rheum 2003;48(5): 1315–25.
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 2001; 25(4):402–8.
16. Martin I, Jakob M, Schafer D, Dick W, Spagnoli G, Heberer M. Quantitative analysis of gene expression in human articular cartilage from normal and osteoarthritic joints. Osteoarthritis Cartilage 2001;9(2):112–8.
17. Ratcliffe A, Doherty M, Maini RN, Hardingham TE. Increased concentrations of proteoglycan components in the synovial fluids of patients with acute but not chronic joint disease. Ann Rheum Dis 1988;47(10): 826–32.
18. Farndale RW, Sayers CA, Barrett AJ. A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. Connect Tissue Res 1982;9(4):247–8.
19. Redini F, Galera P, Mauviel A, Loyau G, Pujol JP. Transforming growth factor beta stimulates collagen and glycosaminoglycan biosynthesis in cultured rabbit articular chondrocytes. FEBS Lett 1988;234(1):172–6.
20. Luyten FP, Hascall VC, Nissley SP, Morales TI, Reddi AH. Insulin-like growth factors maintain steady-state metabolism of proteoglycans in bovine articular cartilage explants. Arch Biochem Biophys 1988; 267(2):416–25.
21. Barry F, Boynton RE, Liu B, Murphy JM. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res 2001;268(2): 189–200.
22. Rayan V, Hardingham T. The recovery of articular cartilage in explant culture from interleukin-1 alpha: effects on proteoglycan synthesis and degradation. Matrix Biol 1994;14(3):263–71.
23. Lefebvre V, Li P, de Crombrugghe B. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. EMBO J 1998;17(19):5718–33.
24. Benya PD, Padilla SR, Nimni ME. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. Cell 1978; 15(4):1313–21.
25. Stokes DG, Liu G, Coimbra IB, Piera-Velazquez S, Crowl RM, Jimenez SA. Assessment of the gene expression profile of differentiated and dedifferentiated human fetal chondrocytes by microarray analysis. Arthritis Rheum 2002;46(2):404–19.
26. Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. Mol Cell Biol 1997;17(4):2336–46.
27. Hardingham T, Tew S, Murdoch A. Tissue engineering: chondrocytes and cartilage. Arthritis Res 2002; 4(Suppl 3):S63–8.
28. Bang OS, Kim EJ, Chung JG, Lee SR, Park TK, Kang SS. Association of focal adhesion kinase with fibronectin and paxillin is required for precartilage condensation of chick mesenchymal cells. Biochem Biophys Res Commun 2000;278(3):522–9.
29. Bridgewater LC, Lefebvre V, de Crombrugghe B. Chondrocyte-specific enhancer elements in the Col11a2 gene resemble the Col2a1 tissue-specific enhancer. J Biol Chem 1998;273(24):14998–5006.
30. Zhang P, Jimenez SA, Stokes DG. Regulation of human COL9A1 gene expression. Activation of the proximal promoter region by SOX9. J Biol Chem 2003; 278(1):117–23.
31. Sekiya I, Tsuji K, Koopman P, Watanabe H, Yamada Y, Shinomiya K, *et al*. SOX9 enhances aggrecan gene promoter/enhancer activity and is up-regulated by retinoic acid in a cartilage-derived cell line, TC6. J Biol Chem 2000;275(15):10738–44.
32. Qi WN, Scully SP. Extracellular collagen modulates the regulation of chondrocytes by transforming growth factor-beta 1. J Orthop Res 1997;15(4): 483–90.
33. Larson CM, Kelley SS, Blackwood AD, Banes AJ, Lee GM. Retention of the native chondrocyte pericellular matrix results in significantly improved matrix production. Matrix Biol 2002;21(4):349–59.
34. Graff RD, Kelley SS, Lee GM. Role of pericellular matrix in development of a mechanically functional neocartilage. Biotechnol Bioeng 2003;82(4): 457–64.
35. van Osch GJ, van der Veen SW, Buma P, Verwoerd-Verhoef HL. Effect of transforming growth factor-beta on proteoglycan synthesis by chondrocytes in relation to differentiation stage and the presence of pericellular matrix. Matrix Biol 1998;17(6):413–24.
36. Zeng L, Kempf H, Murtaugh LC, Sato ME, Lassar AB. Shh establishes an Nkx3.2/Sox9 autoregulatory loop that is maintained by BMP signals to induce somitic chondrogenesis. Genes Dev 2002;16(15): 1990–2005.
37. Zehentner BK, Dony C, Burtscher H. The transcription factor Sox9 is involved in BMP-2 signaling. J Bone Miner Res 1999;14(10):1734–41.
38. Brew CJ, Andrew JG, Boot-Handford R, Hardingham TE. Increased collagen I expression in osteoarthritic cartilage suggests some loss of normal chondrocyte phenotype. Trans Orthop Res Soc 2004;50:940.
39. Farrell CM, Lukens LN. Naturally occurring antisense transcripts are present in chick embryo chondrocytes simultaneously with the down-regulation of the alpha 1 (I) collagen gene. J Biol Chem 1995;270(7): 3400–8.
40. Tsuda M, Takahashi S, Takahashi Y, Asahara H. Transcriptional co-activators CREB-binding protein and p300 regulate chondrocyte-specific gene expression via association with Sox9. J Biol Chem 2003; 278(29):27224–9.
41. Smits P, Li P, Mandel J, Zhang Z, Deng JM, Behringer RR, *et al*. The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. Dev Cell 2001; 1(2):277–90.
42. Smits P, Dy P, Mitra S, Lefebvre V. Sox5 and Sox6 are needed to develop and maintain source, columnar, and hypertrophic chondrocytes in the cartilage growth plate. J Cell Biol 2004;164(5):747–58.
43. Burton-Wurster N, Liu W, Matthews GL, Lust G, Roughley PJ, Glant TT, *et al*. TGF beta 1 and biglycan, decorin, and fibromodulin metabolism in canine cartilage. Osteoarthritis Cartilage 2003;11(3): 167–76.